Molecular Ecology Notes (2005) **5**, 496–498 doi: 10.1111/j.1471-8286.2005.00968.x

PRIMER NOTE

Isolation and characterization of microsatellite loci from the entomopathogenic hyphomycete, *Paecilomyces fumosoroseus*

C. DALLEAU-CLOUET,*¶ N. GAUTHIER,*† A.-M. RISTERUCCI,‡ M.-C. BON§ and J. FARGUES*¶
*Centre de Biologie et de Gestion des Populations, ¶Institut National de la Recherche Agronomique (INRA), †Institut de Recherche pour le Développement (IRD), CS30016, 34988 Montferrier sur Lez, France, ‡UMR 1096 Centre de Coopération Internationale en Recherche Agronomique pour le Développement, TA40/03, 34398 Montpellier Cedex 5, France, §European Biological Control Laboratory, USDA-ARS, CS90013, 34988 Montferrier sur Lez, France

Abstract

Nine microsatellite markers were isolated from the entomopathogenic haploid fungus *Paecilomyces fumosoroseus*. Genetic diversity was assessed in 26 *P. fumosoroseus* isolates originated from the whitefly *Bemisia tabaci* collected in various geoclimatic areas. Eight loci were polymorphic with an observed number of alleles ranging from two to six. The loci differentiated some isolates and group of isolates according to their geographical location, showing promise for the study of gene flow. All loci failed to give clear amplifications in *P. fumosoroseus* isolates from hosts other than *B. tabaci*. These microsatellite markers provide powerful tools for ecological, epidemiological, and population genetic studies.

Keywords: genetic diversity, host insect, hyphomycetes, microsatellites, *Paecilomyces fumosoroseus*

Received 24 December 2004; revision received 21 January 2005; accepted 11 February 2005

Extensive surveys for natural enemies of the whitefly *Bemisia tabaci* (Gennadius), which is considered as a major insect pest in field and greenhouse crops worldwide, revealed the entomopathogenic fungus *Paecilomyces fumosoroseus* (Wize) Brown and Smith (Deuteromycotina: Hyphomycetes) to be the most common pathogen (Lacey *et al*. 1996). *Paecilomyces fumosoroseus* is being commercialized as an environmentally safe myco-insecticide, but its release as an endemic or an exotic biocontrol agent in various geographical environments generates the need for tools valuable in its population genetics. Recently, restriction fragment length polymorphism–internal transcribed spacer (RFLP–ITS) analysis and ITS sequencing data of various *P. fumosoroseus* isolates from different geographical and host insect origins, have gathered lineages into three distinct clusters, one being strictly associated with the insect host *Bemisia* spp. (Fargues *et al*. 2002). Knowledge about the population genetic structure, especially the gene flow between *P. fumosoroseus* populations associated to *B. tabaci*, was limited due to a lack of valuable markers. Only a few microsatellite markers have been isolated in fungi and particularly in entomopathogenic hyphomycetes (Enkerli *et al*. 2001; Rehner & Buckley 2003). Here, we isolated and characterized nine microsatellite loci from 26 *P. fumosoroseus* isolates (*Pfr*) originated from the host *B. tabaci* and tested them on eight isolates from five other insect host species also collected in various geoclimatic areas.

Correspondence: N. Gauthier, Fax: (+33)499623345; E-mail: nathalie.gauthier@ensam.inra.fr

Isolates were selected from both INRA (Montpellier, France) and USDA-ARS (Ithaca, NY) collections of entomopathogenic fungal cultures. Monoconidial cultures and DNA extraction (QIAGEN DNeasy Plant Mini Kit) were conducted as described in Fargues *et al*. (2002). A microsatellite-enriched library was constructed from the total genomic DNA of the isolate *Pfr46*, following the method of Billote *et al*. (1999). Approximately 5 µg of genomic *Pfr46* DNA were restricted to completion with *Rsa*I (Invitrogen). The 350–1500 bp fraction of the digested DNA was ligated to self-complementary adaptors Rsa21 (5′-CTCTTGCTTACGCGTGGACTA-3′) and phosphorylated Rsa25 (5′-TAGTCCACGCGTAAGCAAGAGCACA-3′) (Eurogentec) by T4 DNA ligase (Invitrogen).

Polymerase chain reaction (PCR) products obtained after amplification with Rsa21 primer were purified and

were allowed to hybridize to $(GT)_8$ and $(CT)_8$ 5′biotin-labelled probes (Eurogentec). Labelled probes were subsequently bound to streptavidin-coated magnetic particles (Promega) as described by the manufacturer. The enriched microsatellite fragments were ligated into pGEM-T easy vector (Promega) used to transform XL1-Blue supercompetent cells (Stratagene) as indicated by the supplier.

Recombinant colonies were randomly selected and cloned inserts were amplified with Rsa21 primer. The PCR products were then transferred on Hybond N+ nylon membranes (Amersham) which were hybridized at 56 °C with [γ^{32}P] dATP end-labelled $(GA)_{15}$ and $(GT)_{15}$. Of these recombinant clones, 89.6% (172/192) contained a microsatellite sequence. Sequencing was performed on a random samples of 48 clones using the universal T7 primer and the BigDye Terminator Cycle Sequencing Kit (Amersham), and run on an Applied Biosystems 3730 XL™ DNA analyser (Genome Express).

Nine primer pairs were designed using OLIGO software (version 3.3; National BioSciences) from flanking microsatellite regions containing pure arrays of at least 11 repeats for dinucleotide motives and seven repeats for the tetranucleotide motif.

The PCR amplifications from the selected 34 *Pfr* isolates were carried out in a total volume of 10 μL. Each reaction consists of: 2 μL of genomic *Pfr* isolate DNA (5–7 ng), 1 μL of 10× QIAGEN buffer (with 1.5 mM $MgCl_2$), 2 μL Buffer Q (QIAGEN), 0.4 μL of 1.67 mM d(CGT)TP, 1.2 μL of 50 μM dATP, 0.01 μL of [α^{33}P]-ATP, 0.5 μL of each primer (10 μM), and 0.4 units of *Taq* polymerase (QIAGEN).

The PCR reactions followed a touchdown procedure (Don *et al*. 1991). After an initial denaturing step of 4 min at 94 °C, 10 cycles were performed each consisting of 45 s of denaturation at 94 °C, 30 s at the annealing temperature (T_a) from 58 and 65 and decreasing by 1 °C at each succeeding cycle, and 45 s elongation at 72 °C. Additional 26 cycles were run consisting of 45 s at 94 °C, 30 s at the lower T_a from 48 and 55, and 45 s at 72 °C. A final elongation step was performed at 72 °C for 10 min.

Polymorphism was resolved by running denatured PCR products on a 6% denaturing polyacrylamide gel and by visualizing the fragments by autoradiography. Allele sizes were assigned by comparison with the reference cloned allele of which the exact size was previously determined through sequencing.

Polymorphism was then assessed on the allelic diversity observed at each of the nine loci among the 26 *Pfr* isolates originated from *B. tabaci* (Table 1). The number of alleles per polymorphic locus ranged from two to six for eight loci; one locus being monomorphic. Particular allelic patterns differentiated specific groups of isolates originating from the same country (i.e. Pakistan) or different ones (i.e. India vs. Nepal). Some examples are depicted in Table 2. These microsatellite loci were successfully amplified from all the 26 isolates originated from *B. tabaci*. In contrast, the eight *Pfr* isolates from insect hosts other than *B. tabaci*, namely two Lepidoptera (two isolates from *Mamestra brassicae* and two from *Ostrinia nubilalis*), two Coleoptera (two isolates from *Melolontha melolontha* and one from *Diaprepes abbreviatus*), and an Hemipteran (one isolate from *Antiteuchus*

Table 1 Characteristics of the nine microsatellite loci in the 26 *Paecilomyces fumosoroseus* isolates originated from *Bemisia tabaci* collected in various geographical areas

Locus	Primer sequence (5′–3′) (F: forward; R: reverse)	T_a (°C) range	Repeat motif in clone	Size of cloned allele (bp)	No. of alleles	Allele size range (bp)	GenBank Accession no.
PfrBtA08	F: CTTGTAATCTGTGCGTATGTA R: CTATTAGAAGAAGCGGGAG	60–50	$(GT)_{14}$	259	4	245–259	BV211293
Pfr*BtA10*	F: CTTCATAGGTCTGGACTTGC R: GAAATCAGCGCCAGCTC	60–50	$(CA)_{11}$	71	2	71–77	BV211294
PfrBtB02	F: CGACTCGTCACCACGCAG R: ACTTGTCCGCATCATGCAC	60–50	$(GT)_{20}$	176	6	150–180	BV211295
PfrBtB04	F: GTTGGTGCGGTGTGAGAG R: ATGTAAGGCGATTGGCAG	58–48	$(GT)_{15}$	134	2	126–132	BV211296
PfrBtB07	F: GGAGATATGTGGAGAGTTGA R: CATTATAGCAGCGAACCA	60–50	$(GT)_{15}$	273	3	273–277	BV211297
PfrBtD01	F: CTCGTCATCCAAGGAGAGTC R: GCAATACAGGAACAAGATGC	58–48	$(CA)_{12}$	263	4	261–289	BV211298
PfrBtD05	F: GGCAAGTCACCAGATACGTC R: GGATATGATGAAGCCCTCG	60–50	$(CA)_{12}$	199	4	182–199	BV211299
PfrBtD11a	F: GCGAATCTCGTCTTCACC R: CACTGGCACACGACACTC	65–55	$(GAGT)_7$	109	1	109	BV211300
PfrBtD11b	F: GAGTGTCGTGTGCCAGTG R: GCTATGTGCGTGCTAGATG	65–55	$(GT)_{16}$	85	4	83–93	BV211301

T_a = Annealing temperature («touchdown» PCR), no. of alleles = number of alleles observed.

Table 2 Detailed allelic patterns observed for the eight polymorphic loci described previously in a subset (17) of the 26 *Paecilomyces fumosoroseus* isolates from *Bemisia tabaci* collected in different geographical locations (*n* = number of isolates). The numbers provided in the table are the allele sizes (pb)

	Allelic pattern			
	Pakistan			
Polymorphic locus	*n* = 2	*n* = 5	India *n* = 5	Nepal *n* = 5
PfrBtA08	245	259	247	259
PfrBtA10	71	71	71	71
PfrBtB02	180	180	168	172
PfrBtB04	126	132	126	126
PfrBtB07	273	275	275	277
PfrBtD01	289	263	261	289
PfrBtD05	182	199	197	186
PfrBtD11b	83	85	93	83

tripterus) failed to give clear amplifications, suggesting the main role of the host in the fungus differentiation.

These data emphasized a high level of genetic diversity within the mitosporic fungus *P. fumosoroseus* as previously reported using random amplified polymorphic DNA (RAPD) and vegetative compatibility as genetic markers (Tigano-Milani *et al*. 1995; Cantone & Vandenberg 1998), and confirmed the clear relationship between *P. fumosoroseus* and the host species, *B. tabaci* (Fargues *et al*. 2002). These microsatellite markers provide powerful tools to distinguish isolates and group of isolates in fungal populations originated from *B. tabaci* and from different geographical areas. It may represent an efficient method to investigate the epidemiological potential related to the targeted environments and the interactions between biological control strains and the indigenous populations of *P. fumosoroseus*.

References

Billote N, Lagoda PJL, Risterucci A-M, Baurens F-C (1999) Microsatellite-enriched libraries: applied methodology for the development of SSR markers in tropical crops. *Fruits*, **54**, 277–288.

Cantone FA, Vandenberg JD (1998) Intraspecific diversity in *Paecilomyces fumosoroseus*. *Mycological Research*, **102**, 209–215.

Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Research*, **19**, 4008.

Enkerli J, Widmer F, Gessler C, Keller S (2001) Strain specific microsatellite markers in the entomopathogenic fungus *Beauveria brongniartii*. *Mycological Research*, **102**, 250–256.

Fargues J, Bon M-C, Manguin S, Couteaudier Y (2002) Genetic variability among *Paecilomyces fumosoroseus* isolates from various geographical and host insect origins based on the rDNA-ITS regions. *Mycological Research*, **106**, 1066–1074.

Lacey LA, Fransen JJ, Carruthers RI (1996) Global distribution of naturally occurring fungi of *Bemisia*, their biology and use as biological control agents. In: *Bemisia 1995: Taxonomy, Biology, Damage, Control and Management* (eds Gerling D, Mayer R), pp. 401–433. Intercept, Andover, UK.

Rehner SA, Buckley EP (2003) Isolation and characterization of microsatellite loci from the entomopathogenic fungus *Beauveria bassiana* (Ascomycota: Hypocreales). *Molecular Ecology Notes*, **3**, 409–411.

Tigano-Milani MS, Honeycutt J, Lacey LA, Assis R, McClelland M, Sobral WS (1995) Genetic variability of *Paecilomyces fumosoroseus* isolates revealed by molecular markers. *Journal of Invertebrate Pathology*, **65**, 274–282.